

# Yeast homolog of a cancer-testis antigen defines a new transcription complex

Elena Kisseleva-Romanova<sup>1,5</sup>, Raffaele Lopreiato<sup>1</sup>, Agnès Baudin-Baillieu<sup>1,6</sup>, Jean-Claude Rousselle<sup>2</sup>, Laila Ilan<sup>1</sup>, Kay Hofmann<sup>3</sup>, Abdelkader Namane<sup>2</sup>, Carl Mann<sup>4</sup> and Domenico Libri<sup>1,\*</sup>

<sup>1</sup>Centre National de la Recherche Scientifique, Centre de Genetique Moleculaire, Gif sur Yvette, Paris, France, <sup>2</sup>Institut Pasteur, Génopole, Plate-Forme de Protéomique, Paris Cedex, France, <sup>3</sup>Miltenji Biotech GmbH, Koeln, Germany and <sup>4</sup>Biochemistry Department, F Edward Hébert School of Medicine, USUHS, Bethesda, MD, USA

We have isolated a new yeast gene (*PCC1*) that codes for a factor homologous to human cancer-testis antigens. We provide evidence that Pcc1p is a new transcription factor and that its mutation affects expression of several genes, some of which are involved in cell cycle progression and polarized growth. Mutation of Pcc1p also affects the expression of *GAL* genes by impairing the recruitment of the SAGA and Mediator co-activators. We characterize a new complex that contains Pcc1p, a kinase, Bud32p, a putative endopeptidase, Kae1p and two additional proteins encoded by ORFs *YJL184w* and *YMLO36w*. Genetic and physical interactions among these proteins strongly suggest that this complex is a functional unit. Chromatin immunoprecipitation experiments and multiple genetic interactions of *pcc1* mutants with mutants of the transcription apparatus and chromatin modifying enzymes underscore the direct role of the complex in transcription. Functional complementation experiments indicate that the transcriptional function of this set of genes is conserved throughout evolution.

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## Introduction

Transcription of approximately 10% of the yeast genome occurs in a cell-cycle-dependent manner (Spellman *et al*, 1998). For almost half of these genes, the peak of transcription takes place around START (Spellman *et al*, 1998; Horak

*et al*, 2002) when cells enter a new cycle and start budding. Thus, it is not surprising that an efficient transcriptional machinery is required for the passage through START. Indeed, mutation of several factors of the transcriptional apparatus leads to defects in START and the accumulation of unbudded cells (Jahng *et al*, 1988; Prendergast *et al*, 1990; Drebot *et al*, 1993; Walker *et al*, 1997; Koch *et al*, 1999). Modulation of gene expression is also critical for yeast conjugation. The response to mating pheromone in *Saccharomyces cerevisiae* involves the transcriptional activation of roughly 200 genes (Roberts *et al*, 2000), which ultimately determines cell-cycle arrest in the G1 phase and the formation of mating projections.

Transcription starts when specific activators bind to their cognate sites on the DNA and recruit co-activator complexes. Co-activators promote the formation of the preinitiation complex (PIC) containing general transcription factors (GTFs) and RNA polymerase II (RNAPII) at the core promoters. This event determines the initiation of transcription, which is followed by elongation and termination.

Transcription through chromatin requires the action of chromatin modifying and chromatin remodeling factors. Covalent modifications of histones include phosphorylation, ubiquitylation, methylation and acetylation (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Acetylation of histone N-terminal tails is generally associated with transcriptional activity and histone acetyltransferases (HAT) are recruited to promoters by transcriptional activators. Although several histone deacetylases (HDAC) contribute to transcriptional repression by interacting with specific DNA-binding repressors, recent reports suggest that in some instances it is the dynamic interplay between acetylation and deacetylation that affects transcriptional activity (Wang *et al*, 2002; De Nadal *et al*, 2004). Post-translational modification of transcription factors can also ultimately lead to their degradation (Muratani and Tansey, 2003; Gillette *et al*, 2004; Muratani *et al*, 2005) although the mechanism of proteolysis-mediated transcriptional control is still unclear.

Here, we report the first functional characterization of new *S. cerevisiae* proteins involved in transcription. These proteins belong to a complex that we have named the EKC (Endopeptidase-like Kinase Chromatin-associated) complex. Mutation of EKC complex components leads to several defects in cell cycle progression and polarized growth that can be ascribed to a defective transcriptional response. We provide evidence that the EKC complex impacts transcription of *GAL* genes and is required for the efficient recruitment of transcriptional co-activators. Remarkably, the complex contains a protein kinase, Bud32p and a putative metalloprotease/ATPase, Kae1p. We show that the conserved zinc-binding domain of Kae1p is essential for its function, suggesting a proteolytic activity for the EKC complex that might be related to its role in transcription. Several subunits of the complex are conserved from archae to man and we provide evidence that these sequence similarities reflect a

\*Corresponding author. Centre National de la Recherche Scientifique, Centre de Genetique Moleculaire, 91190 Gif sur Yvette, Paris, France. Tel.: +33 1 69823809; Fax: +33 1 69823877; E-mail: libri@cgm.cnrs-gif.fr

<sup>5</sup>Present address: Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU, UK

<sup>6</sup>Present address: Institut de Génétique et Microbiologie, Université Paris-Sud, 91405 Orsay, France

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functional homology. Together, these data point to the existence of a new function in transcription that has deep evolutionary roots.

## Results

### Isolation of the *PCC1* gene

We performed a screen to isolate suppressors of a splicing defect due to a U to A mutation in the fifth nucleotide of the U1snRNP particle (U1-5A; Seraphin *et al*, 1988), which leads to a cold-sensitive phenotype. Four such suppressors contained genomic fragments overlapping an intergenic region between *MLP1* and *YKR096w*. Close inspection of the region revealed the presence of a short putative ORF containing an intron with a noncanonical splice site (GUAAAGU). While this work was in progress, a putative ORF was identified based on phylogenetic analysis and assigned the name *YKR095-A* (Brachet *et al*, 2003). A small protein of about 10 kDa derived from this ORF could be identified both by chromosomal tagging and mass spectrometry analysis (see below). We called this gene *PCC1* (Polarized growth Chromatin-associated Controller 1, see below). Overexpression of *PCC1* on a multicopy plasmid, or expression of its intronless version integrated on the chromosome, was sufficient to suppress the cold-sensitive phenotype, but did not affect splicing efficiency (data not shown). Rather, splicing of the *PCC1* intron was strongly and specifically affected by the U1-5A mutation, presumably due to the destabilizing effect of an A-A mismatch present in the duplex between U1-5A snRNA and the 5' splice site of the *PCC1* intron (data not shown). This indicates that splicing of the *PCC1* intron is the main limiting factor for growth in the U1-5A strain.

### *Pcc1p* is required for normal cell cycle progression and mating projection formation

*PCC1* is not essential, but *pcc1* null cells grow very slowly at all temperatures and are thermosensitive at 34°C (data not shown). We thus constructed a thermosensitive mutant allele, *pcc1-4*, that we integrated at the *PCC1* genomic locus to facilitate phenotypic analyses. Visualization of DAPI stained *pcc1-4* cells after a shift to the nonpermissive temperature (37°C) showed a marked increase in the fraction of unbudded cells with a single nucleus compared to the WT (Supplementary Figure S1) and a concomitant decrease in the fraction of small to medium budded cells (S-G2) and anaphase cells. These results show that *pcc1-4* cells are defective for bud formation at the nonpermissive temperature. In contrast, the fraction of large-budded cells with two separated nuclei was not significantly reduced compared to the WT, suggesting the existence of an additional defect in exit from mitosis or cytokinesis. Microtubule staining of *pcc1-4* cells at the nonpermissive temperature with GFP-Tub1 indicated that the mitotic spindle had disassembled in these cells, suggesting that they had undergone mitosis and were delayed for cytokinesis/cell separation (data not shown).

A parallel FACS analysis of *pcc1-4* cells at the nonpermissive temperature (Supplementary Figure S1B) indicated an increase in the population of cells with a 1C DNA content, most likely representing unbudded cells, and a decrease in S-phase cells. Consistent with cytological observations, we did not observe complete disappearance of the population with a 2C DNA content. The existence of two defects at the

G1/S and M/G1 transitions was confirmed by FACS analysis of alpha factor and nocodazole-synchronized cells and colony-forming assays indicated that *pcc1-4* cells maintained high viability after 8 h of incubation at the restrictive temperature (data not shown).

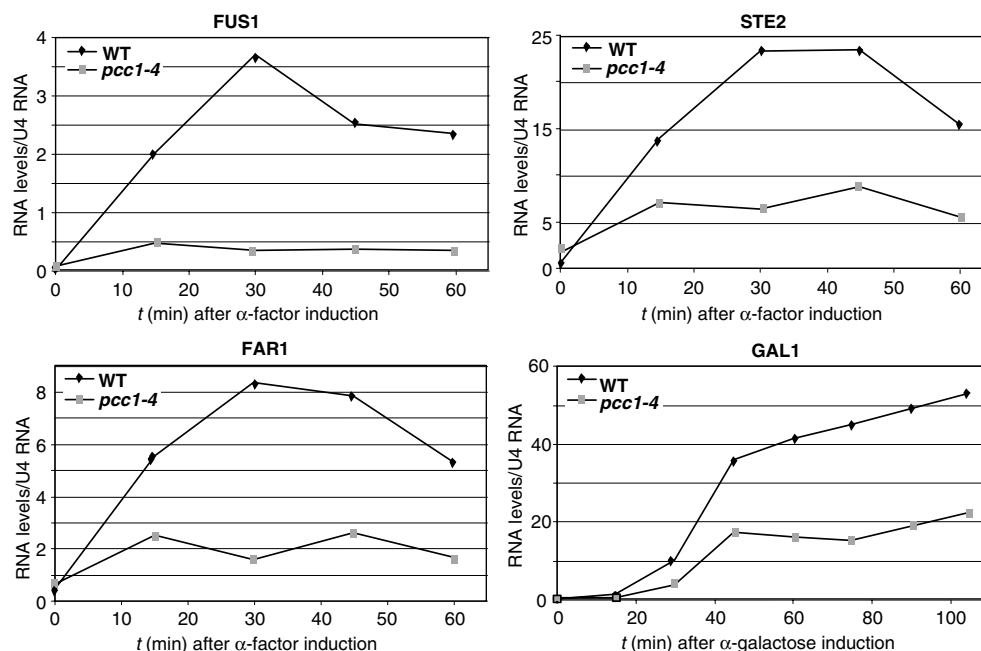
Treating *MATa* cells with alpha pheromone blocks cells in the G1 phase and induces mating projections. Remarkably, pheromone-treated *pcc1-4* cells accumulated largely as unbudded cells without mating projections at the nonpermissive temperature (Supplementary Figure S1C). Unbudded *pcc1-4* cells at 37°C accumulated faster in the presence compared to the absence of alpha factor (data not shown), indicating that *pcc1-4* cells arrest division in response to pheromone, but they are defective in pheromone-induced morphogenesis.

### *Pcc1p* is required for normal gene expression

To gain mechanistic insight into the function of *Pcc1p*, we first analyzed its role in the mating pheromone response that is characterized by the activation of a complex transcriptional program. We analyzed by real time RT-PCR the expression of a set of genes induced by alpha factor to assess the integrity of the transcriptional response in *pcc1-4* cells. *FUS1*, *FAR1* and *STE2* are strongly induced after alpha-factor exposure. As shown in Figure 1, the kinetics of induction of the three genes was significantly affected by the *pcc1-4* mutation. To determine whether mutation of *Pcc1p* impacts specifically the expression of alpha-factor inducible genes, we performed the same analysis for the *PMA1*, *GAL1* and *HSP104* genes that are, respectively, constitutively expressed, induced by galactose and by heat shock. Mutation of *Pcc1p* resulted in a significant decrease of *GAL1* expression after galactose induction (Figure 1), but did not affect *HSP104* induction and *PMA1* mRNA levels (data not shown).

To monitor transcriptional activity more directly, we analyzed RNAPII occupancy in the *pcc1-4* mutant at the nonpermissive temperature by chromatin immunoprecipitation (ChIP) experiments. Mutation of *Pcc1p* significantly affects RNAPII density on *STE2*, *FUS1* and *GAL1* genes, and the lower levels of RNAPII were evenly distributed along the ORFs (Figure 2A and data not shown).

Formation of the transcription PIC requires the association of specific activators with upstream activating sequences (UAS), which promote the interaction of co-activators and TBP (Spt15p) with the nearby promoters. Activation of galactose-inducible genes requires the sequential recruitment of the SAGA and Mediator co-activators by the DNA-bound Gal4p activator (Bryant and Ptashne, 2003; Bhaumik *et al*, 2004). To identify the step of transcription that is affected by mutation of *Pcc1p*, we analyzed the recruitment of Gal4p activator as well as SAGA and Mediator components (respectively Gcn5p and Rgr1p) and TBP (Spt15p) to the *GAL1* gene in a *pcc1-4* genetic background. As shown in Figure 2B, binding of the Gal4p activator was not affected by mutation of *Pcc1p*. However, recruitment of the co-activators SAGA (Gcn5p) and Mediator (Rgr1p) were strongly affected. As expected, recruitment of TBP was also strongly diminished both at the *GAL1* gene (Figure 2B) and the *STE2* gene when the latter was induced by alpha factor (data not shown). The reduced presence of these proteins (and of RNAPII, Figure 2A) at the transcriptionally active *GAL1* gene does not reflect a decrease in their steady state levels in the *pcc1-4*



**Figure 1** Real-time RT-PCR analysis of mRNA levels produced by  $\alpha$ -factor-inducible and galactose-inducible genes in wild type and *pcc1-4* mutant cells. Wild type and *pcc1-4* mutant cells were preincubated for 3 h at 37°C before the addition of  $\alpha$ -factor for analysis of *FUS1*, *FAR1* and *STE2* transcripts, or in raffinose-containing medium before the addition of galactose for analysis of *GAL1* transcripts. Samples were taken at the indicated time points. Signals (arbitrary units) were normalized to *U4* snRNA, the abundance of which was not affected by mutation of Pcc1p.

background at the nonpermissive temperature as verified by Western blot analysis (Supplementary Figure S2A).

We also analyzed the recruitment of the Ste12p activator to the promoter of the *FAR1* gene, whose transcription is sensitive to Pcc1p (Supplementary Figure S2B). In contrast to the Gal4p activator, binding of Ste12p has been shown to increase upon activation of transcription (Ren *et al*, 2000). In both wild type and *pcc1-4* cells at the nonpermissive temperature, binding of Ste12p was increased by a very similar factor upon pheromone exposure, further confirming that mutation of Pcc1p does not affect signaling or activator binding.

Together, these results indicate that Pcc1p is required for the efficient transcription of genes induced by alpha factor and galactose. Pcc1p function is dispensable for the binding of the Gal4p and Ste12p activators, but is necessary for the efficient recruitment of TBP and the SAGA and Mediator co-activators at the *GAL1* promoter. These results indicate that *pcc1* mutants are affected at an early step of transcription activation that precedes TBP recruitment and PIC assembly. Finally, they also strongly suggest that the polarized growth defects observed in *pcc1* mutants are due to an impairment of the transcriptional response.

#### **Pcc1p associates with the chromatin of RNAPII transcribed genes**

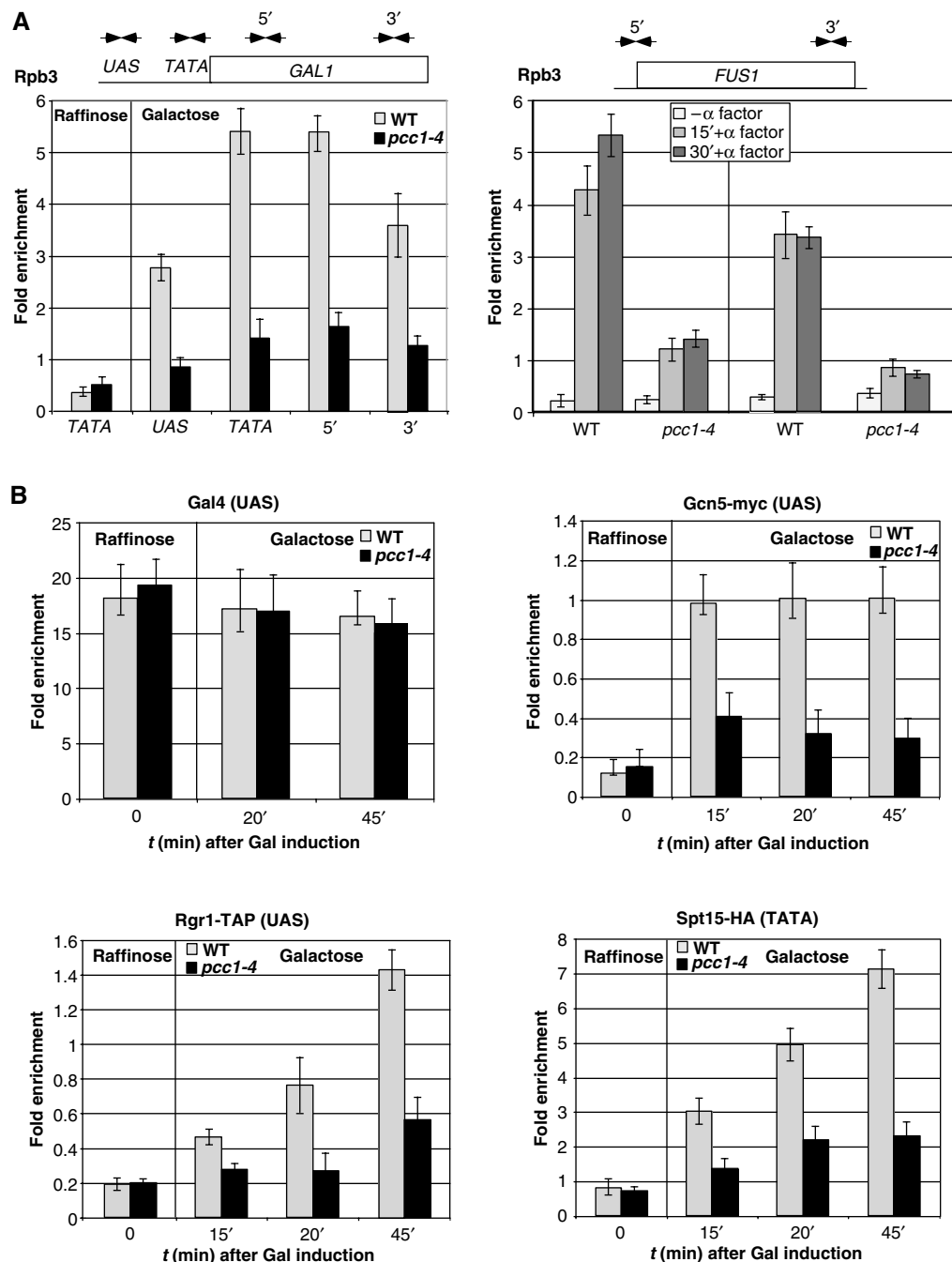
To rule out possible indirect effects, we asked whether Pcc1p is recruited to the DNA of its target genes, and whether recruitment occurs in a transcription-dependent manner. To this end, we performed ChIP analysis with yeast cells expressing a TAP-tagged form of Pcc1p. Pcc1-TAP is functional, as this strain was only slightly affected for growth at 37°C. *MAT a* cells were grown at 30° and aliquots were taken before and after (5 and 15 min) alpha factor addition. Real-time PCR

analysis of DNA co-immunoprecipitated with Pcc1-TAP indicates that it associates strongly with *STE2*, *FUS1*, *FAR1* and *FUS3* genes upon transcriptional induction (Figure 3A), but poorly with an intergenic nontranscribed region (Supplementary Figure S3). Note that the Pcc1-TAP ChIP signal is comparable to the signals observed for Rgr1-TAP and Gcn5-myc (Figure 2B). We also analyzed Pcc1-TAP recruitment to *GAL1* and *GAL10* genes under nonactivating (raffinose-containing medium), activating (galactose-containing medium), and repressing (glucose-containing medium) conditions. As shown in Figure 3B, the Pcc1-TAP ChIP signal was low in nonactivating conditions, increased upon galactose induction, and rapidly dropped to background levels by glucose-induced transcriptional repression. Pcc1-TAP was evenly distributed along the whole *GAL1-10* (Figure 3B) and *STE2* (data not shown) transcription units. Finally, Pcc1p bound in a transcription-dependent manner to the heat-inducible *HSP104* and to the constitutively expressed *PMA1* genes, although we did not observe an affect of the *pcc1-4* mutation on the production of mRNAs from these genes (data not shown), suggesting that the Pcc1p-dependency for different genes is not related to its differential recruitment.

Taken together, these data indicate that Pcc1p is recruited to several independently regulated genes (alpha-factor, galactose- and stress-induced genes) in a transcription-dependent manner.

#### **PCC1 interacts genetically with genes encoding components of the transcription machinery and chromatin modifying enzyme**

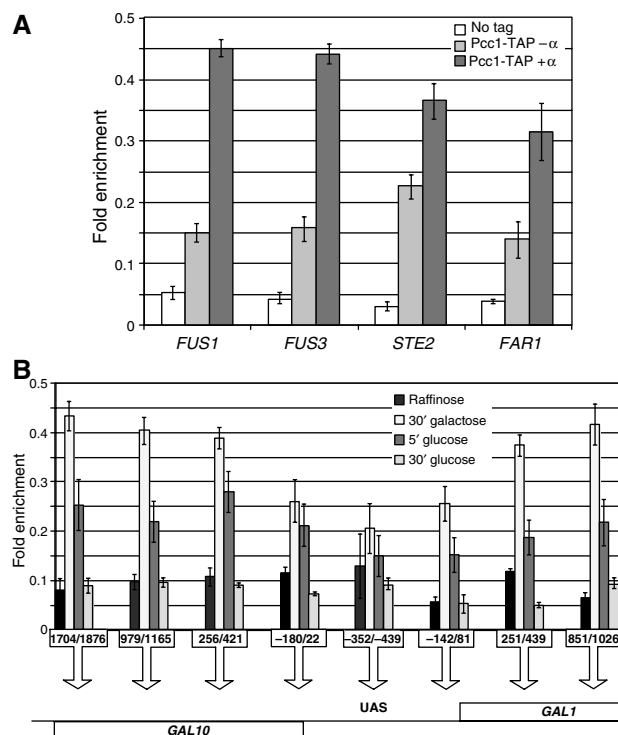
To further test the implication of Pcc1p in transcription, we sought genetic interactions between *pcc1-4* and mutants of the transcription apparatus. Mutation of Pcc1p severely affected growth when associated with mutants of RNAPII



**Figure 2** (A) RNAPII recruitment to transcriptionally active genes is affected in the *pcc1-4* mutant. Wild type and *pcc1-4* mutant cells were preincubated for 3 h at 37°C before addition of galactose or  $\alpha$ -factor. Chromatin was immunoprecipitated with anti-Rbp3p antibodies. (B) Recruitment to the *GAL1* locus of the Gal4p activator, SAGA (Gcn5p), Mediator (Rgr1p), and TBP (Spt15p) in *pcc1-4* mutant cells. Antibodies against Gal4p, myc and HA epitopes (all from Santa Cruz Biotech) were used, respectively, to probe Gal4p, Gcn5p-myc and Spt15-HA. IgG Sepharose was used to immunoprecipitate Rgr1-TAP. In (A, B), immunoprecipitated DNAs were analyzed by real-time PCR with primers for *GAL1* and *FUS1* genes, respectively (the position of primers is schematically shown) and expressed relative to input DNA (arbitrary units). Averages and standard deviations were obtained from three independent experiments.

holoenzyme components such as Rpb1p, Rpb4p and Rpb9p (Supplementary Figure S4). In the latter case, the genetic interaction is particularly strong as the *pcc1-4 rpb9 $\Delta$*  double mutant is inviable at 34°C while the two single mutants grow reasonably well at this temperature. The *pcc1-4* mutation also severely affected growth when combined with *rad3-6.4*, a transcription-defective allele of *RAD3* (Jensen *et al*, 2001, 2004) that is a component of the GTF TFIIH.

Efficient transcription in the context of a chromatin template requires the recruitment to promoters of co-activators that possess chromatin-modifying activities. The SAGA and NuA4 complexes are transcriptional co-activators endowed with histone acetylase activity borne, respectively, by the Gcn5p and Esa1p subunits. Double mutants *pcc1-4 gcn5 $\Delta$*  and *pcc1-4 esa1-1851* (as *ESA1* is essential, we used a thermo-sensitive allele in this case) were strongly affected in growth



**Figure 3** Pcc1p associates with the chromatin of transcriptionally active genes. (A) *PCC1-TAP* and isogenic untagged cells were grown at 30°C to early exponential phase before addition of  $\alpha$ -factor. Cells were harvested for ChIP preparations after 15 min of incubation with  $\alpha$ -factor. Immunoprecipitated and input (total) DNA were analyzed with primers located at the 5' coding regions of the *FUS1*, *FUS3*, *STE2* and *FAR1* genes. Averages and standard deviations were obtained from three independent experiments. (B) *PCC1-TAP* and isogenic untagged cells were grown at 30°C in raffinose to early exponential phase before addition of galactose. After 30' of galactose induction, growth was shifted to glucose-containing media to shutoff transcription of *GAL* genes. Cells were harvested for ChIP analysis at the indicated time points in the three different growth conditions. Immunoprecipitated DNAs were analyzed by real-time PCR with primers spanning the entire *GAL1*–*GAL10* region and expressed relative to input DNA (arbitrary units). Immunoprecipitations were performed with IgG-Sepharose. The high level of Pcc1p occupancy at the UAS region in raffinose growth conditions was not consistently observed. Values are averages from duplicate points.

compared to single mutants, further underscoring the relationships between Pcc1p and efficient transcription. This is also consistent with the finding (reported above) that Pcc1p integrity is required for efficient Gcn5p recruitment and suggests that Pcc1p might collaborate with additional co-activators (i.e. NuA4) in the regulation of gene expression.

The histone deacetylase Rpd3p has been shown to affect gene expression both positively and negatively (Kadosh and Struhl, 1998; Bernstein *et al*, 2000; Kurdistani *et al*, 2002; Robyr *et al*, 2002; De Nadal *et al*, 2004; Carrozza *et al*, 2005). Deletion of *RPD3* in a *pcc1-4* context led to a strong synthetic growth phenotype (Supplementary Figure S4). We also constructed a *pcc1-4 sin3Δ* double mutant, as Sin3p is part of the histone deacetylase B (HDB) complex that contains Rpd3p and is required for its activity (Kadosh and Struhl, 1997; Kasten *et al*, 1997). *pcc1-4 sin3Δ* cells grew poorly (data not shown), which confirms the requirement for Rpd3p deacetyl-

ase when Pcc1p function is impaired. Finally, deletion of *HDA1*, encoding the catalytic component of the histone deacetylase A (HDA) (Robyr *et al*, 2002), or deletion of *HOS2*, belonging to the same class of HDAC as Rpd3p, did not affect growth of a *pcc1-4* mutant (data not shown).

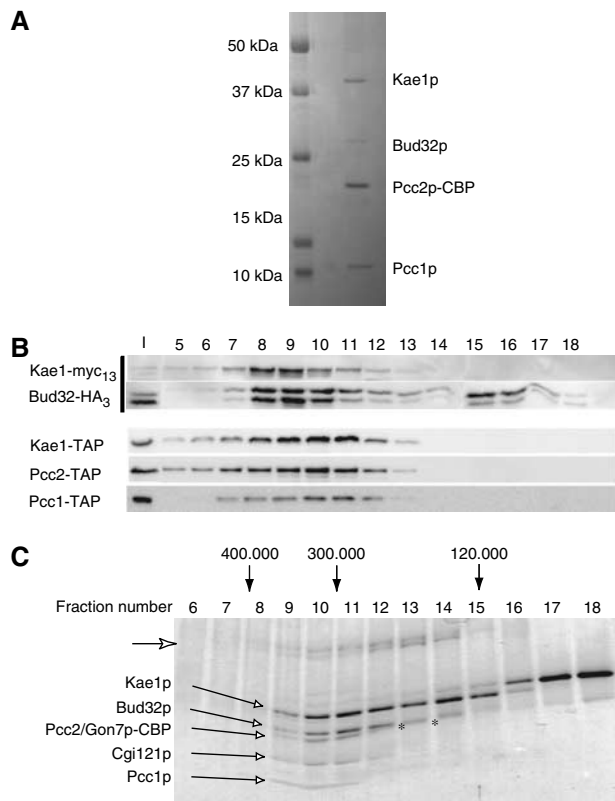
Together these results underscore a role for Pcc1p in transcription, and might suggest a function related to chromatin remodeling/modifying.

### Isolation and characterization of the Pcc1p-containing complex

To identify proteins interacting with Pcc1p, we purified TAP-tagged Pcc1p (Rigaut *et al*, 1999) and analyzed the associated complex by mass spectrometry (data not shown). In addition to Pcc1p, the purified complex contained Kae1p, Bud32p and a small protein encoded by ORF *YJL184w* that was assigned the temporary designation Gon7p and which we propose to name Pcc2p. TAP-tag purification of the Pcc2p-associated complex followed by mass spectrometric peptide analysis confirmed the co-purification of these four proteins (Figure 4A). Gel filtration analysis of whole-cell extracts containing tagged subunits confirmed that the four proteins elute in a fraction corresponding to a molecular weight of approximately 300 000 (Figure 4B). Bud32p was also found to elute in a second peak that does not contain any of the other subunits. To prove the existence of a single complex containing all the subunits, we purified Pcc2p/Gon7p from cell extracts by affinity chromatography. The eluted complex was then fractionated on a Superdex 200 sizing column. The simultaneous elution of the four subunits in the same fractions (Figure 4C) indicates that they indeed form a single complex with an apparent molecular weight of approximately 300 000. In this experiment, we also identified by mass spectrometry a fifth protein that is the product of ORF *YML036w*, the yeast homologue of human Cgi121p. A distinct complex eluting in fractions 13–15 (apparent MW of approximately 130 000) was also identified that only contains Kae1p and Pcc2p/Gon7p (Figure 4C; note that only Pcc2p/Gon7p was identified by a combined MS and MS/MS analysis in the band marked with an asterisk, see the legend of Figure 4). The smaller complex was not evident in the gel filtration of whole-cell extracts, so it may result from dissociation of Pcc1p, Cgi121p and Bud32p during the purification of the larger complex.

Interaction of Kae1p, Bud32p, Gon7p and Cgi121p was also reported in a large-scale analysis of yeast complexes (Ho *et al*, 2002; Gavin *et al*, 2006). The Bud32p–Kae1p, Pcc1p–Pcc2p, and Pcc1p–Kae1p interactions were also observed by two-hybrid analysis (our unpublished results; Lopreiato *et al*, 2004). Pcc2p appears to have orthologues only in fungi and no known functional domains were found in its sequence. Kae1p, on the other hand, is universally conserved and is similar in sequence to a family of zinc-dependent endoproteases (Mellors and Lo, 1995; Lopreiato *et al*, 2004). Finally, Bud32p is a serine/threonine kinase homologous to human PRPK, a factor that has been shown to bind and phosphorylate p53 (Abe *et al*, 2001; Facchin *et al*, 2003). We named this complex the EKC to reflect these characteristic features and its association with transcribed chromatin (see above).

Although *KAE1* and *PCC2* are strictly nonessential, *pcc2Δ* and *kae1Δ* cells grow poorly at all temperatures (data not shown). Therefore, we generated temperature sensitive



**Figure 4** Isolation and characterization of the EKC complex. (A) TAP purification of the Pcc2p-associated complex. Co-purifying proteins were identified by mass spectrometry. (B) Gel filtration of whole-cell extracts from the indicated strains. Western blots of the indicated fractions were probed with anti-myc, anti-HA and PAP (peroxydase-anti peroxidase) complex. Note that the first two Western blot stripes derive from a doubly tagged Bud32-HA/Kae1-myc strain, which most likely accounts for the slightly different hydrodynamic properties of the complex compared to the single TAP-tagged Kae1p, Pcc1p and Pcc2p/Gon7p strains. I: Input. (C) Gel filtration of the affinity-purified EKC complex. The Pcc2-TAP complex was bound to IgG-Sepharose and eluted by cleavage with the TEV protease. The eluate was subjected to Superdex 200 gel filtration and the proteins in individual fractions were visualized by Coomassie blue staining after 5–20% SDS-PAGE. The identity of the bands was confirmed by mass spectrometry. The band marked with \* in fractions 13–14 was identified as Pcc2p/Gon7p-CBP by combined MS and MS/MS analysis and does not appear to contain Bud32p. These two proteins comigrate in some gels (see fraction 12 and data not shown). The open arrows indicate two proteins that have been identified as the Hsp70 chaperones Ssa2p and Ssb1p. The protein found in fractions 17–18 is an unidentified contaminant. Apparent molecular weights are indicated by filled arrows.

mutants of both genes and integrated them at their respective genomic loci. These mutants have a cell cycle phenotype that is similar to that of *pcc1* cells (data not shown) and were inviable when combined with the *pcc1-4* allele, suggesting that either mutant alone partially compromises the function of the EKC complex and that the latter is required for cell viability (Supplementary Figure S5). Pcc2p/Gon7p and Kae1p are nuclear (data not shown) and can be crosslinked to the DNA of alpha factor induced genes and *GAL1* (Supplementary Figure S5 and data not shown), suggesting that the whole complex is recruited to genes during transcription. Together, these data strongly suggest that the EKC complex is a functional unit.

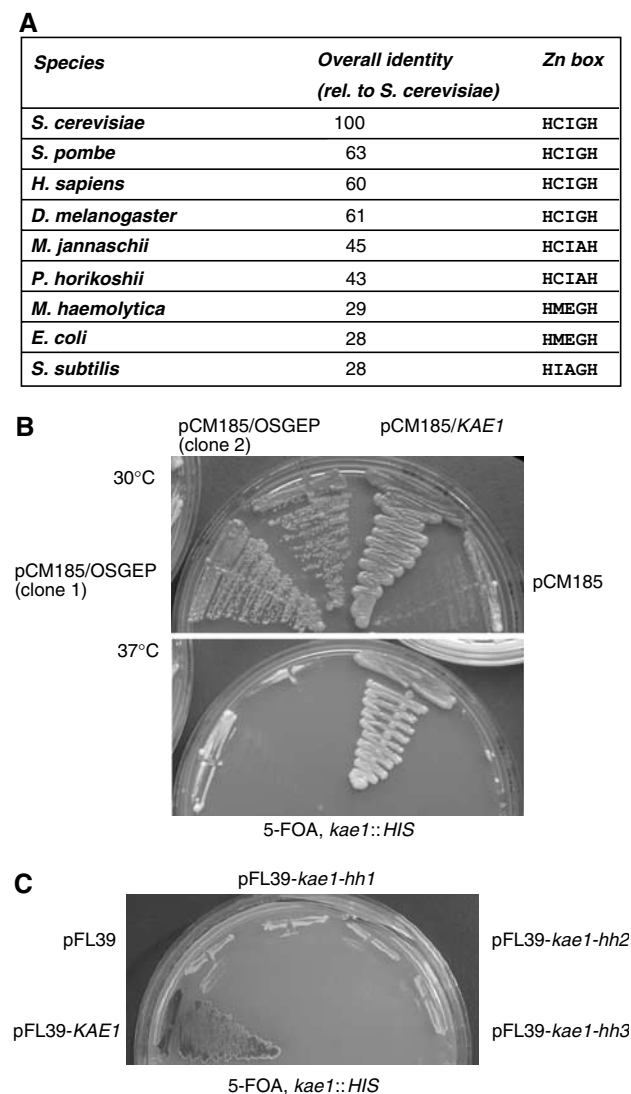
### Conservation of the EKC complex

Comparison of the Pcc1p protein sequence with several databases revealed that this gene belongs to a very large family of proteins conserved from archaeobacteria to man (Supplementary Figure S6). Three genes coding for putative homologous proteins are present in the human genome. CTAG1/NY-ESO1 and CTAG2/NY-ESO2 belong to the family of cancer-testis antigens and are specifically expressed in several human tumors of different origin and in normal testes and ovaries (Chen *et al*, 1997, 1998; Jager *et al*, 1998; Stockert *et al*, 1998). A third gene, ESO3, codes for a protein that is ubiquitously expressed (Faranda *et al*, 1996; Alpen *et al*, 2002) and is similar to NY-ESO1 and NY-ESO2. Expression of ESO3 in yeast did not restore normal growth to a *pcc1* null strain (data not shown). However, sequence comparison of the PCC1 family allowed the identification of two conserved domains of unknown function that we named Pcc1 Homology Box 1 (PHB1) and 2 (PHB2) (Supplementary Figure S6). Within PHB1 8 residues were almost universally conserved among the different family members. Deletion of PHB1 or mutation of its highly conserved residues led to inactivation of the protein, indicating that this region is required for Pcc1p function or stability (Supplementary Figure S6). Interestingly, replacing the N-terminal domain with the corresponding regions from the human CTAG1/NY-ESO1 or ESO3 sequences partially restored Pcc1p function (Supplementary Figure S6), which suggests that the Pcc1p gene and its human homologues share at least some functions.

Kae1p is also extremely well conserved in archae and eukaryotes (Figure 5A, see also Lopreiato *et al*, 2004), which is indicative of an important role that has deep evolutionary roots. To assess whether phylogenetic conservation implied a functional relationship, we cloned the putative human *KAE1* homologue, the OSGEP gene, in a yeast expression vector. Interestingly, OSGEP partially complemented the growth defects of the yeast *kae1Δ* mutant (Figure 5B), indicating that Kae1p function is conserved through evolution and strongly suggesting that OSGEP is a functional homologue of *KAE1*. Together with the reported finding that Bud32p and its human homologue PRPK are functionally related (Facchin *et al*, 2003), these data strongly suggest conservation of EKC complex function in higher eucaryotes.

### The zinc-binding motif of Kae1p is required for viability

Sequence similarities indicate that Kae1p is a putative endopeptidase of the M22 family of metalloproteases (Mellors and Lo, 1995; Lopreiato *et al*, 2004). Similar enzymes are also present in bacteria, and an enzymatic activity has been experimentally demonstrated for the *Pasteurella haemolytica* enzymes (Abdullah *et al*, 1991). The catalytically essential residues of these proteins have not been identified, but the putative zinc-binding domain is highly conserved from bacteria to man (Figure 5A) and is believed to be required for the activity of these metalloproteases. We mutated the two conserved histidines to random residues and analyzed several mutants for growth. All mutations at these two positions completely inactivated Kae1p function (Figure 5C). Together, these results indicate that the metal binding domain (and possibly the endopeptidase activity) are required for the essential function of Kae1p.



**Figure 5** Kae1p contains a highly conserved and functionally critical Zn-binding endoprotease motif. (A) Overall identity of Kae1p homologs from several species to the *S. cerevisiae* sequence. The sequence of the putative Zn-binding domain is shown in the right column. (B) The human OSGEP gene partially complements the *kae1Δ* mutant. Two independent clones bearing the OSGEP gene expressed from a Tet promoter-based centromeric plasmid were introduced into a *kae1::HIS*, *pCM188(URA)*-*KAE1* shuffle strain and assayed for growth on 5-FOA plates at 30 and 37°C as indicated. Human OSGEP partially complements the growth defect of the *kae1::HIS3* mutant at 30°C, but not 37°C. (C) Mutation of the two histidines in the putative Zn-binding is incompatible with Kae1p function. Three different histidine mutants (*kae1-hh1*, *kae1-hh2* and *kae1-hh3*) were introduced into the *kae1::HIS*, *pCM188(URA)*-*KAE1* shuffle strain and assayed for growth on 5-FOA plates at 30°C. Clones *kae1-hh1*, *kae1-hh2* and *kae1-hh3* bear respectively the H176L/H180G, H176V/H180V and H176V/H180T mutations.

## Discussion

### The EKC complex is involved in transcription

In this paper, we report the first characterization of a novel and highly conserved complex of proteins with a crucial role in transcription. *pcc1* mutant cells are defective for normal cell cycle progression and are unable to fully respond to pheromone. We show that transcriptional defects are likely to be responsible for these phenotypes.

The response to pheromone involves the transcriptional induction of genes required for mating behavior, including cell cycle arrest and the formation of mating projections. We found that transcripts derived from several pheromone-inducible genes, such as *FUS1*, *STE2* and *FAR1* are significantly decreased in *pcc1* mutant cells upon the shift to the nonpermissive temperature, which was paralleled by the decreased RNAPII and TBP occupancy at the genomic loci, indicating the transcriptional nature of the defect. Finally, the implication of Pcc1p (and presumably the whole EKC complex) in transcription is likely to be direct, as we demonstrated by ChIP experiments showing that Pcc1p, Kae1p and Pcc2p (this report) and Bud32p (Lopreiato *et al*, in preparation) are recruited to several genomic loci upon transcriptional induction. The ChIP signal we observed for Pcc1-TAP at *GAL1* was comparable to the signal observed for components of the transcriptional co-activators SAGA and Mediator. This notion is also strongly supported by genetic interactions between *pcc1-4* and several mutants of the transcriptional and chromatin-modifying machinery.

The transcriptional role of the EKC complex is not restricted to pheromone-inducible genes, but also extends to the galactose-inducible *GAL1* gene. Preliminary DNA microarray experiments also identified a set of genes that are affected in the *pcc1-4* mutant at the nonpermissive temperature. Among these we found, as expected, several pheromone-inducible genes (*FUS1*, *FUS3*, *BAR1*), but also genes required for cytokinesis (*SCW1*, *CWP1*, *HOF1*, *CTS1* and *EGT2*), and polarized cell growth (*CLN2*, encoding a G1 cyclin), which might explain at least part of the phenotypes observed in *pcc1-4* cells.

### The EKC complex is required for efficient recruitment of transcriptional co-activators

ChIP analyses indicate that Pcc1p is required for efficient recruitment of SAGA and Mediator co-activators (or at least their Gcn5p and Rgr1p components) to the promoter of the *GAL1* gene. As defective recruitment of these complexes affects TBP (Spt15p) and RNAPII recruitment (which we observe), it is likely that a transcriptional defect in *pcc1-4* cells lies upstream of PIC formation and downstream of Gal4p binding at the level of SAGA recruitment to the *GAL1* promoter and/or the stabilization of this interaction, which is known to precede binding of the Mediator (Bryant and Ptashne, 2003; Bhaumik *et al*, 2004).

Although we have not detected Gal4p or SAGA or Mediator components in EKC preparations by mass spectrometry, it is possible that these interactions are transient and do not survive the purification procedure. Consistent with this notion, Gal4p, the SAGA component Taf6p, and the Mediator component Med7p were isolated in the same large-scale two-hybrid screen that also identified Pcc2p and Kae1p as prey using Pcc1p as a bait (our unpublished data). It is possible that these interactions contribute to the overall network of interactions that recruits and/or stabilizes the factors required for PIC formation. Recent work from the Tansey and Workman laboratories (Lee *et al*, 2005) has unveiled a role for the 19S proteasome regulatory particle in promoting the interaction of the SAGA co-activator with transcriptional activators, perhaps by remodeling the SAGA complex. Interestingly, in a large-scale study of yeast protein complexes (Ho *et al*, 2002), Bud32p and Kae1p were found to

coprecipitate with five subunits (Rpt1p, Rpt3p and Rpn1p, Rpn5p and Rpn6p) of the 19S regulatory component of the proteasome. This physical interaction between members of the EKC complex and the 19S proteasome, and the observation that both promote SAGA interaction with the *GAL* genes, further underscore a direct role for the EKC in transcription and might underlie a common mechanism of action.

A role in transcriptional activation for the EKC is generally consistent with the genetic interactions we observed. The strong genetic interaction observed between *PCC1* and the NuA4 histone acetylase *ESA1* might indicate that Pcc1p (and the EKC complex) are also required for efficient recruitment of NuA4, and possibly of other co-activators, to their target genes. A strong genetic interaction was also found with the *RPD3* HDAC. Although HDACs are generally associated with repression of gene expression, a direct role for Rpd3p in the activation of several stress-induced genes was recently described (De Nadal *et al*, 2004), suggesting the possible existence of common targets with the EKC complex. Whether the genetic interactions with histone acetylases/deacetylases imply a synergistic relationship in gene activation through a chromatin-modifying function is a matter for further investigations.

The transcription-dependent distribution of Pcc1p as measured by ChIP is not restricted to the promoter regions of the genes tested. The reason for this is presently unclear, although it is not uncommon that the distribution of ChIP signals for several transcription or nontranscription factors do not reflect the main (or the most visible) role of the factor. As for the 19S particle of the proteasome (Ferdous *et al*, 2001; Gonzalez *et al*, 2002; Gillette *et al*, 2004), it is possible that the distribution of the EKC complex reflects an additional and overlapping role in transcription elongation and/or termination.

### **The components and conservation of the EKC complex**

The EKC complex contains, beside Pcc1p, four additional proteins: Kae1p, Gon7p/Pcc2p, Bud32p and Cgi121. With the exception of Pcc1p these proteins were also found to interact in a recent large-scale analysis (Gavin *et al*, 2006) of protein complexes. We extend here these results and prove by gel filtration analysis that they define a single complex an apparent molecular mass of 300 kDa. Preliminary cryo-EM images of the complex (data not shown) suggest an elongated form, which might explain the discrepancy between the expected and the apparent molecular mass. The physical interaction of these proteins, their genetic interactions and the similar phenotype of thermosensitive mutants indicate that the EKC complex is a functional unit.

Kae1p, Pcc1p and Bud32p are universally conserved from archaeobacteria to man, with a strikingly high level of identity (roughly 60%) between the human homologue of Kae1p (OSGEP) and the yeast protein. Interestingly, the Bud32p and Kae1p homologues in archae are fused in the same polypeptide (Lopreiato *et al*, 2004). Based on the functional complementation data reported here and in another report for PRPK, the human homologue of Bud32p (Facchin *et al*, 2003), it is likely that a related complex exists in most or all eukaryotes and archae with a similar transcription-related function. Consistent with this notion, it was shown that PRPK is able to bind and phosphorylate p53, thus activating p53-dependent transcription (Abe *et al*, 2001; Facchin *et al*, 2003).

It is tempting to speculate that the function of the CTAG1 and/or CTAG2 cancer-testis antigens (Chen *et al*, 1998) is also linked to the transcription process and that their derepression in tumors may be causally related to oncogenesis.

### **What is the mechanism of action of the EKC complex?**

One of the most interesting features of the EKC complex is a putative Zn-binding endopeptidase activity associated with the Kae1 subunit. Although proteolytic activity has not been directly demonstrated for Kae1p, we showed that its putative zinc-binding domain, which is conserved in the bacterial proteases, is required for its function *in vivo*. A role for a proteolytic activity in transcription regulation is not unprecedented. The proteolytic activity of the 26S proteasome is required for its role in transcription termination, although the targets remain elusive (Gillette *et al*, 2004). Similarly, degradation of a transcriptionally engaged form of Gal4p was recently shown to be required for the production of translatable RNAs (Muratani *et al*, 2005). Thus, the EKC complex might impact transcription through its proteolytic activity, although the targets and mechanism of action remain to be identified. Sequence similarity searches have also revealed the presence of an Hsp70 fold in the Kae1p family, leading to the suggestion that this protein is an ATP-dependent protease with chaperone-like activity (Aravind and Koonin, 1999). It is thus possible that a chaperone activity is responsible for the transcriptional role of Kae1p independently of its (putative) endopeptidase function. In this perspective, the EKC complex might impact transcription through an ATPase function in a way that would be analogous to that proposed for the 19S particle of the proteasome (Ferdous *et al*, 2001; Ezhkova and Tansey, 2004; Lee *et al*, 2005).

While this work was in progress, Downey *et al* (2006) identified subunits of the EKC complex, which they named KEOPS, as being involved in telomere maintenance. The authors provide evidence that the Bud32p and Cgi121p proteins are required for telomere uncapping and single-strand DNA accumulation in a *cdc13-1* capping mutant. Deletion of *BUD32* is also shown to affect *de novo* synthesis of telomeres, but neither telomerase recruitment nor its activity in an *in vitro* assay. The authors suggest that Bud32p (and presumably the KEOPS complex) promotes accessibility to the chromosome 3' end, which could explain the seemingly antithetical effects of being required both for telomere synthesis and telomere degradation in a capping mutant.

At this stage, it is unclear whether two distinct functions exist for the EKC/KEOPS complex or whether there is only one primary function that affects both transcription and telomere length. We note that the essential function of the EKC/KEOPS cannot be the maintenance of telomere length and/or the positive regulation of telomerase function as thermosensitive mutants of Pcc1p, Gon7p/Pcc2p and Kae1p stop dividing at the nonpermissive temperature after a few divisions, while loss of telomerase leads to replicative senescence only after 60–80 doublings (Lundblad and Szostak, 1989).

Two recent genome-wide studies (Askree *et al*, 2004; Gatbonton *et al*, 2006) have reported that telomere length is controlled by a large number of genes (estimated at 3% of the yeast genome), the largest fraction of which codes for factors involved in transcription and RNA metabolism (Askree *et al*,

2004; Gatbonton *et al*, 2006). Thus, it cannot be excluded that some unknown consequences of perturbing transcription (or RNA processing/turnover) affects telomere maintenance.

However, we favor the hypothesis that the primary role of Bud32p and the EKC/KEOPS complex relates to the establishment and the maintenance of the structure of chromatin, possibly through an ATP-dependent remodeling function borne by Kae1p. Such a function would be required at telomeres as well as elsewhere in the genome, thus affecting chromosome 3'-end accessibility and transcription.

The characterization of the EKC/KEOPS complex by Downey *et al* (2006), and in this study opens up the exciting perspective of defining the targets and mode of action of a potentially new mechanism to control the expression and the integrity of the genome.

## Materials and methods

Standard methods for construction and manipulation of yeast strains are described in Supplementary methods

### RNA and ChIP analysis

RNAs were prepared and analyzed by real-time RT-PCR (Light-Cycler, Roche as previously described (Libri *et al*, 2002; Jensen *et al*, 2004)). Primers used are listed in Supplementary Table S2. Amplification efficiencies were calculated from serial dilutions for every set of amplification reactions. RNA levels were normalized to the levels of U4 snRNA. As reactions for U4 snRNA have to be diluted to maintain linearity, the ratios are expressed in arbitrary units.

Preparations of chromatin were performed essentially as described (Jensen *et al*, 2004). Immunoprecipitations of 500 µl fractions were performed with saturating amounts of anti-Rpb3p (Neoclone), anti-Gal4 (Santa Cruz Biotech.), anti-myc (9E10, Santa Cruz Biotech.), anti-HA (F-7, Santa Cruz Biotech.) antibodies or with 25 µl of IgG-Sepharose (Amersham). Immunoprecipitated DNA was quantified by real-time PCR (LightCycler), normalized to a dilution of input DNA and expressed as arbitrary units. Amplification efficiencies for every set of primers (Supplementary Table S2) was measured as described above.

### Protein purification and identification

TAP tag purifications of the EKC complex were performed from a yeast whole-cell extract (121, OD<sub>600</sub> of 1.0–1.5), essentially as

described (Rigaut *et al*, 1999). Gel filtration analysis was performed on a Superdex 200 (10/300 mm) column with whole-cell extracts or after the first purification step of the TAP-tag procedure.

Proteins were identified by peptide mass fingerprinting with a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) Voyager DE-STR mass spectrometer (Applied Biosystems, Framingham, MA, USA). The Investigator Progest system (Genomic Solutions) was used for in-gel digestion of specific protein bands with modified porcine trypsin (Promega), and the Investigator ProMS system (Genomic Solutions) was used for purification with Zip-Tip C18 (Millipore) and for loading the sample with the matrix (alpha-Cyano 4-Hydroxy Cinnamic Acid) on the Maldi sample plate.

Monoisotopic masses were assigned using a local copy of the MS-Fit3.2 portion of the Protein Prospector package (University of California, Mass Spectrometry Facility, San Francisco, USA) to search the yeast ORF database. The parameters were set as follows: no restriction on the isoelectric point of proteins. In total, 50 p.p.m. were allowed as the maximum mass error, and one incomplete cleavage per peptide was considered.

Proteins present within the gel-filtered complexes were identified by a combined analysis using MS and MS/MS data (4800 MALDI TOF/TOF Analyser, Applied Biosystems, MDS.SCIEX). The number of precursor peaks for MS/MS acquisition per band and S/N filter were set at 10 and 20 respectively. For searching the yeast ORF database, a local copy of Mascot (Matrix Science, London, UK) was used with a mass tolerance of 50 p.p.m. for MS and 0.3 Da for MS/MS. The database search criterions were based on individual protein ion score (based on MS and MS/MS) and individual ion score (based on MS/MS)  $\geq 95\%$  confidence interval ( $P < 0.05$ ) for unknown proteins searched against yeast ORF database.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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